In The Name Of God

Site-specific PEGylation of an anti-CEA/CD3 bispecific antibody improves its antitumor efficacy

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Introduction

Antibodies have attracted increasing interest as therapeutics due to their unique
biophysical characteristics, such as good solubility, stability, high specificity, and
prolonged plasma concentration.

bispecific antibodies have been developed as a powerful approach to cancer immunotherapy
by engaging immune cells to target cancer cells.

Bispecific antibodies have two different antigen-binding sites:
1)recognizes tumor cells
2)recognizes immune cells, usually T cells or natural killer(NK) cells.

Nb Derived from natural camel heavy-chain only antibodies
single-domain antibodies lack the first constant (CH1) domain and light chain and are consequently referred to as nanobodies or VHHs

Nanobodies are small sized

more stable than c ScFvs or BiTEs (bispecific t-cell engager)
a good scaffold for constructing bispecific antibodies

- S-fab was developed -can bind to diverse epitope-be synthesized in prokaryotic expression systems.
- S-Fab has potent in vitro cytotoxicity
- suppresses cancer progression in in vivo studies
- have a short plasma half-life

To improve the in vivo half-life of proteins:

1) conjugation to polyethyleneglycol (PEG; PEGylation)
2) N-(2-hydroxypropyl) methacryl-amide (HPMA) copolymers
3)proteins (such as albumin)
4) polyglutamic acid and PASylation

Other benefits of PEGylation

reducing the antigenicity and immunogenicity of the conjugated proteins
improving protein solubility
enhancing the proteolytic resistance of therapeutic proteins
decreasing toxicity, and improving the thermal and mechanical stabilities

In this study, we explored thiol site-specific PEGylation
to improve the half-life (t1/2) of the CEA-S-Fab bispecific antibody
, which is an antiCEA/CD3 bispecific S-Fab

A functionalized 20 kDa linear PEG was conjugated to S-Fab.

To retain the properties of CEA-S-Fab, the site for conjugation was designed to be distant to both CEA- and CD3-binding sites.

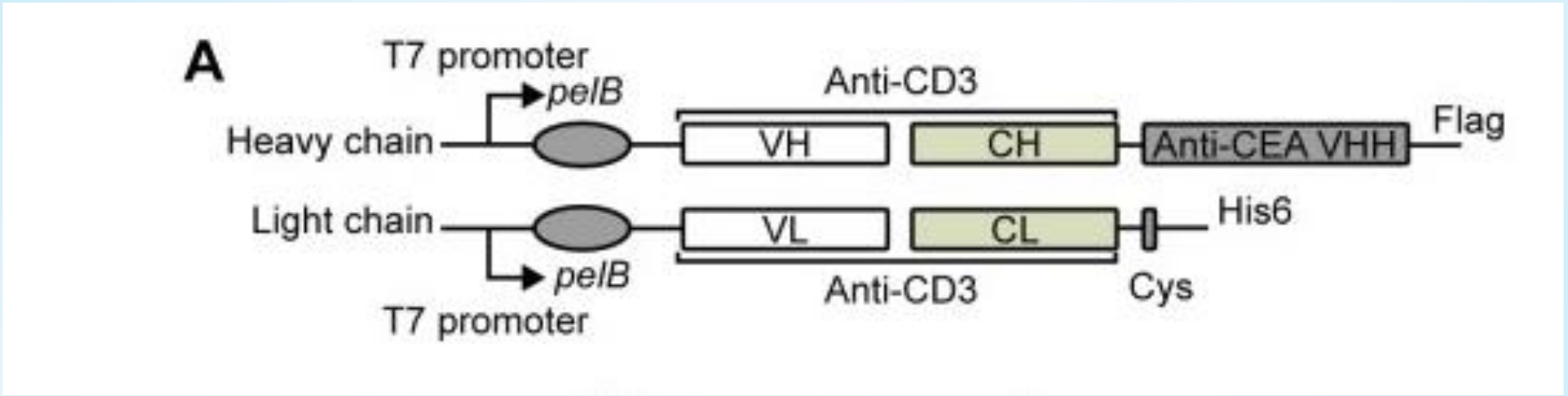
Material and methods

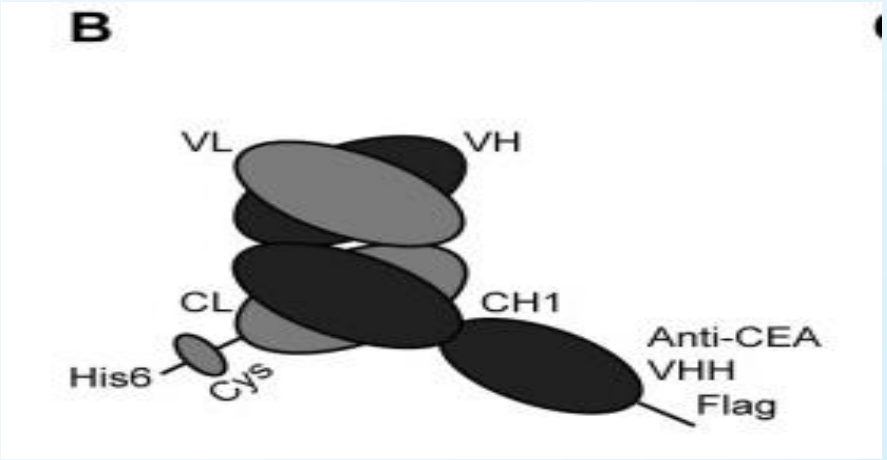
The 20 kDa linear methoxy PEG maleimide (MAL-PEG-OMe; Lot A3115)
Human colon adenocarcinoma cell line LS174T and human breast cancer cell line SKOV3
(NOD–SCID) mice (18–22 g)

Adult male Sprague Dawley (SD) rats(250–300 g) that were used for PK analysis

Methods

1)S-Fab design and protein purification





To produce S-Fab, two plasmids encoding the VH-CH1-VHH and VL-CL polypeptides were co-transformed into BL21 (DE3, codon plus) competent cells with the appropriate antibiotics.

When the absorbance (OD600) of the cell culture reached 0.8
0.2 mM isopropyl-β-d-thiogalactoside (IPTG)was added to induce protein expression.
Cells were cultured at
16°C for another 40 h before harvesting.

periplasmic extraction was performed
by re-suspending the cell pellets in a pre-cooled sucrose solution (20 mM Tris-HCl, pH 7.5; 25% [w/v]sucrose; 1 mM EDTA).

After incubation on ice, the
suspension was centrifuged and the supernatant fraction was collected as the sucrose fraction.

The pellet was re-suspended in a chilled periplasmic solution
(5 mM MgCl2) and centrifuged .
supernatant was gathered as the periplasmic fraction.

The S-Fab was purified from the combined sucrose and periplasmic fractions by a two-step :

1) by immobilized Ni-NTA affinity chromatography

2) by an IgG-CH1affinity matrix

Gel filtration analysis was performed using a Bio-Rad FPLP system

analysis under reducing conditions.

The resulting fractions were visualized by Coomassie blue staining. Protein mark-ers (Lot MWGF200; Sigma-Aldrich Co.) were loaded as standard controls for gel filtration analysis.

2)Conjugation of S-Fab to PEG

- S-fab in 5ml pbs &1 mM tris 2-carboxy ethyl phosphine were mixed and ancubated to obtain reduced s-fab fragment

- To explore the optimal molar ratio of **MAL-PEG-Ome** and **S-Fab** in the PEGylation
- a series of reactions with the molar equivalents of PEG:S-Fab of 0:1, 10:1, 20:1, 40:1, and 60:1.

MAL-PE - OMe was dis-solved in sterile water to obtain a working concentration of 20 mg/mL (1 mM).

- PEGylationof S-Fab was carried out by mixing MAL-PEG- OMe (at the working concentration) with reduced S-Fab and shaking at 22°C for 2 h.

- Result ⟶ samples ⟶ 12%reduced ⟶ SDS-PAGE

  or non reduced

- ⟶ western blot ⟶ transfer polyvinylidene fluride bloking (skimmed milk) ⟶ membrane were incubated with antiflag HRP(1:2000 heavy chain) and anti-His IgG(1:3000 light-chain) in5%skim milk ⟶ after washing with tris-buffered saline &Tween 20 buffer ⟶ secondary Ab (HRP-conjougate IgG1:13000)Incubated with light-chain ⟶ membrane were developed pierce's west pico chemiluminescence

3)Purification of PEG-S-Fab using fast protein liquid chromatography

PEG-S-Fab was purified using an AKTA™ avant25 fast protein liquid chromatography purification system (GE Healthcare Bio-Sciences Corp.) and a Superdex 10/300 GL column at a flow rate of 0.8 mL/min. The column was first equilibrated with two column volumes (CVs) of distilled water and two CVs of PBS before applying the samples. All the collected fractions were analyzed by Coomassie blue and barium iodide complex staining after SDS-PAGE under reducing conditions. The fractions of the purified PEG-S-Fab were pooled together for further studies.

4)Human CD3 T-cell isolation

Human PBMCs were prepared from healthy donors using Ficoll gradient centrifugation as previously described.[8,11] T cells were isolated from PBMCs using an EasySep™ Human CD3 Positive Selection Kit. Isolated T cells were cultured in complete Roswell Park Memorial Institute 1640 medium with 10% FBS and 1% penicillin/streptomycin at 37°C in a 5% CO_2 humidified incubator before cytotoxicity assays.

5)Flow cytometric analysis

1)Detect CEA → cell line LS174T & SKOV3 → centrifugation → washing ice-cold PBS+0.2% BSA → $1\times10_6$ LS174T & SKOV3or $5\times10_5$ TCELL → S-Fab & PEG_sfab incubated on ice for 1h →

washing twice with ice-cold PBS +BSA → Anti-CD3 fluoresceine isothiocyanate (OKT3) as +control forCD3+antigen-binding analysis .

→ Goat antihuman IgG (H +L)-AlexaFluor 488 antibody added . → cells were incubated on ice for 1 h → After washing the cells twice,. → Flow cytometric

6)Immunofluorescence assay

1)LS174T & SKOV3 were plated 30 mm confocal glass bottom dishes

2) Washed with cold PBS three times

3)Fixed cells were incubated with S-fab or peg-s fab + IgG (H+L)AlexaFluor488 antibody for 2 h at 4°C.

4) Cell nuclei were counterstained with 4′,6-diamidino-2-phenyl indole (DAPI).

5) washing with PBS,

6)the sampleswere examined under an scan-ning confocal microscope

7) analyzed using the Olympus FV31S-SW_V2.1 software.

7)In vitro cytotoxicity

1) LS174 & SKOV3 → target cells
2) CD3+ T cell without prior stimulation → effector cells
3) Cytotoxicity assay → 96-well microplate -triplate-seeding 5000 target cell in *100ML media*

4) 6 h incubation an equal volume of CD3+ Tcell add to each well an E:T ratio of 10:1

5)add a series of concentrations (0.033,0.1, 0.33, 1, 3.3, 10, 33, and 100 nM) of S-Fab or PEG-S-Fab

6) After a 72 h incubation, the cell viability was evaluated

8)PK assay to determine the in vivo half-life

1) Specific pathogen free male SD rats (250–300 g) were used for the PK assay.

2). S-Fab , PEG-S-Fab, or a volume equivalent of the vehicle solutionPBS was administered through the caudal vein.

3) 0, 0.5, 1, 2, 4,8, 16, 24, 36, 48, 72, 96, and 144 h after administration

. ➡ Sample blood ➡ orbital vein ➡ using capillary

4)Blood sample ➡ centrifugation ➡ stored at -80

5) S-Fab & PEG-S-Fab in the plasma samples were quantified using ELISA.

6)100 µL aliquot of 6D6 (mouse antihuman IgG Fab antibody; 1.0 µg/mL in PBS) was used to coat each well of a 96-well ELISA microplate for 2 h at 37°C.

7) The wells were blocked with blocking buffer(PBST containing 1% BSA)

8) Samples and standards (100, 80,50, 40, 30, 20, 10, 5, 1, and 0.1 µg/mL) in → blocking buffer → (S-Fab)/PBS 1:10 → important,to avoid matrix effects in the assay

9) 1:3 dilution plasma

10) Sample added in triplicate and incubated

11) each well → washed with PBST → added secondary Ab(mousemonoclonal antiflagM2-peroxidase [HRP] antibody at 1:500 dilutions)per well

12) Five time washed → 100 μL tetramethylbenzidine substrate solution was add to each well

13)Incubation

14) 100 μL of 2 M H_2SO_4 Was added to stop the reaction

15) The absorbance was detected at 450 nm using a Tecan ELISA microplate reader.

16)The serum elimination t1/2 and clearance were calculated with 3P97 PK software

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9)Plasma stability analysis

S-Fab and PEG-S-Fab stabilities were assessed in human fresh plasma over 2 weeks. Briefly, S-Fab and PEG-S-Fab were diluted with human fresh plasma (without platelets), which generated an initial concentration of 100 μg/mL. Simultaneously, a vehicle control with only plasma was established. The samples were incubated at 37°C for 2 weeks. At the time intervals of 0, 24, 48, 72, 96, 168, 264, and 336 h, 40 μL samples were collected and stored directly at −80°C until further analysis. The samples were thawed on ice and centrifuged at 18,500× *g* for 10 min at 4°C. The supernatant was subjected to electrophoresis in 12% reducing SDS-PAGE (5 μL of sample per well). After electrophoresis, a Western blotting assay was performed to analyze the protein levels.

10)In vivo tumor growth inhibition

1)using NOD-SCID mice engrafed subcutane ously with LS174 cell
2)Cells harvested and washed once with PBS and mixed with human PBMCs that were freshly isolated from healthy donors.
3) Mixtures of LS174T cells and human PBMCs were subcutaneously injected into the right flank of NOD–SCID mice
4) One hour after engraftment, S-Fab & PEG-S-Fab or the vehicle control (PBS) were injected intraperitoneally.
5) The tumor volume was measured with calipers in two perpendicular dimensions
6) All data were expressed as the mean ± SEM for each group, and differences between groups were determined by a two-way analysis of variance using the GraphPad Prism 5 soft-ware

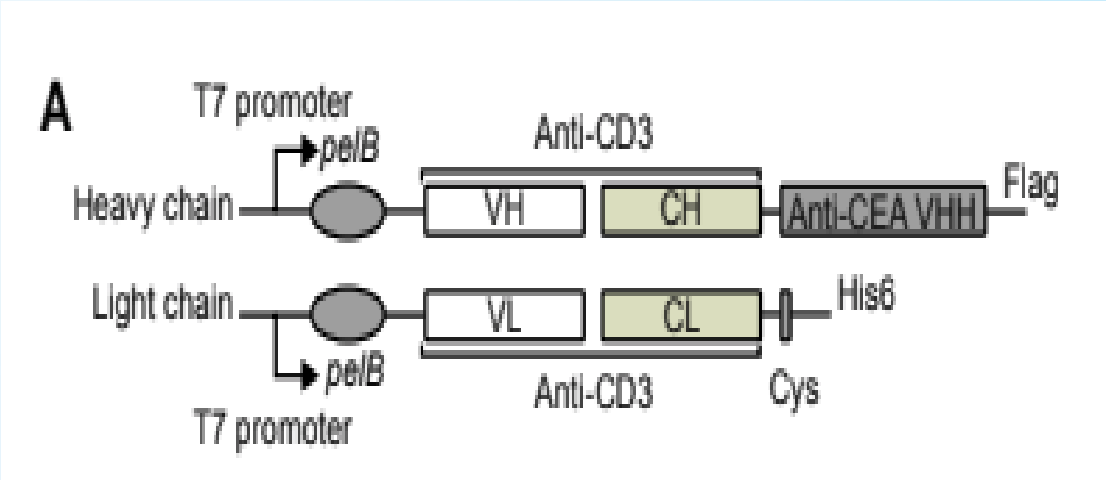
Result

1)Bispecific S-fab construction and purification

- S-fab was constructed with the following specification

- Peptid(CGGGGC) added to C-terminal of anti-CD3 CL-VL

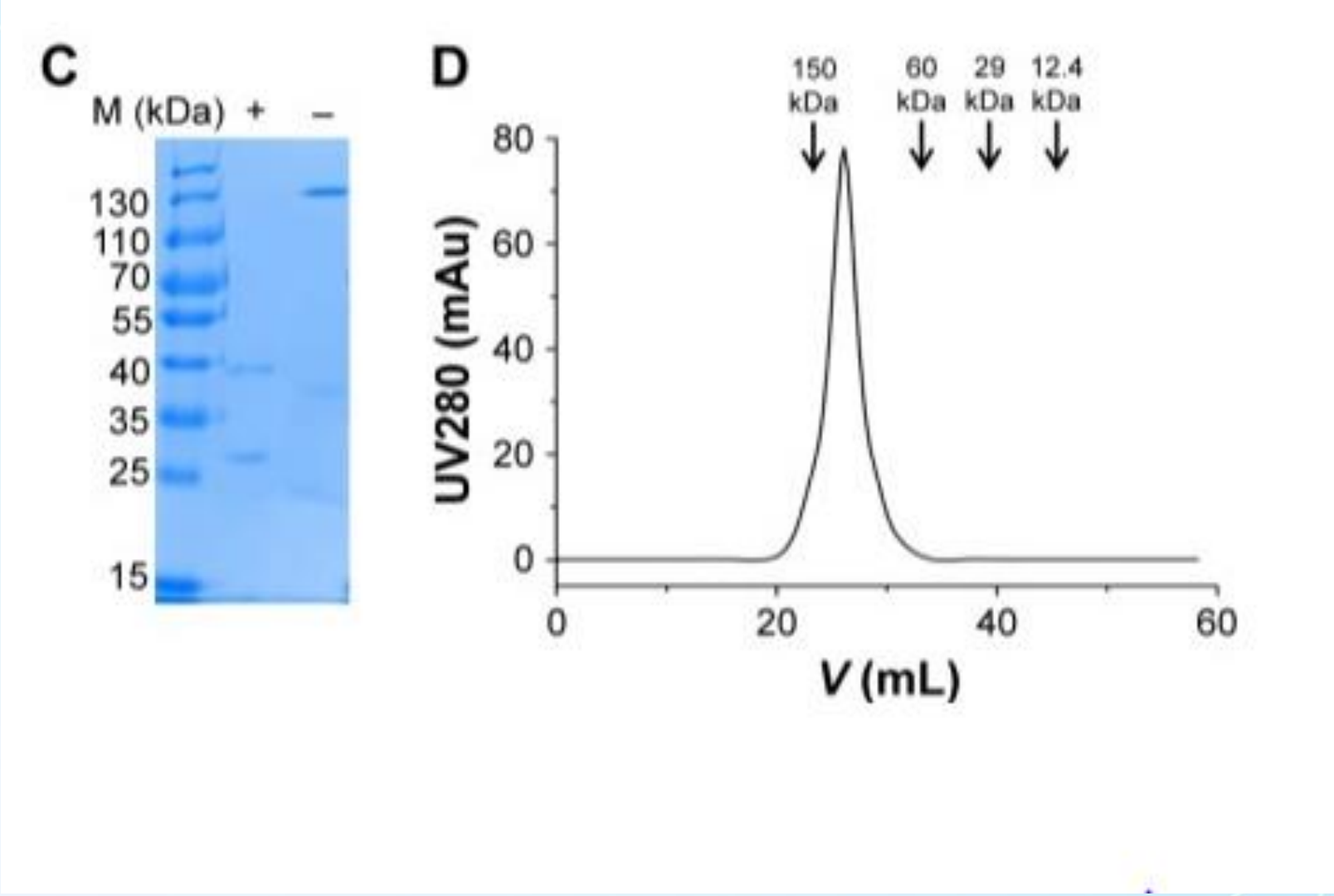
- Cloned into PET26b & PET21a With signal peptide for pelB



- C)
- Ligh chain 25KD
- Heavy chain 40KD

(under reducing condition 2-mecapto ethanol)

A major binding 130KD observed (under nonreducing)= Dimerzation s-fab



d)Gel filtration analysis
Intact s-fab Ab 130KD

2)Conjugation of s-fab to PEG

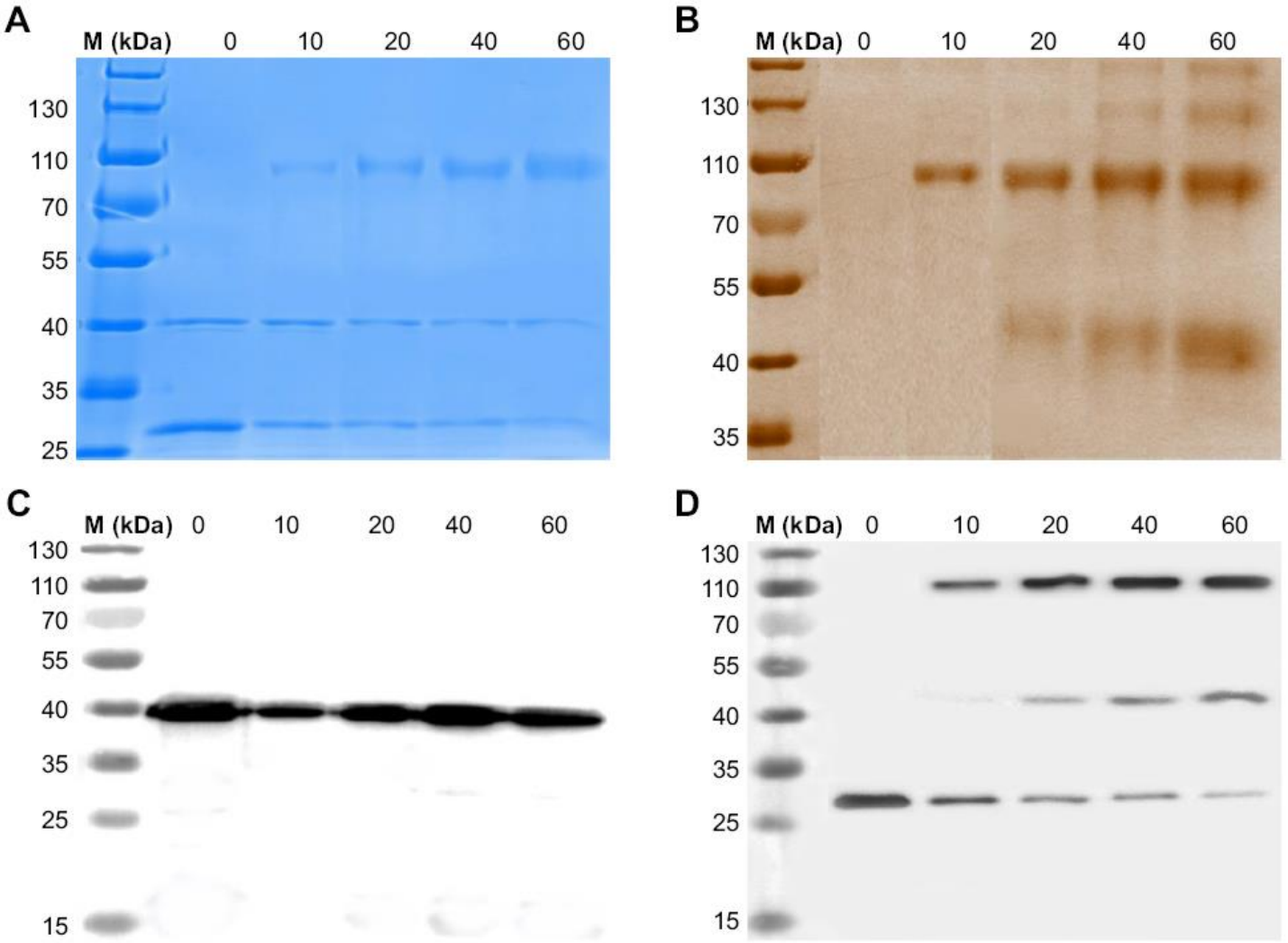


Figure 2 PEGylation of bispecific S-Fab using 20 kDa linear MAL-PEG-OMe.

Notes: S-Fab was reacted with the functionalized PEG at a series of ratios (0:1, 10:1, 20:1, 40:1, and 60:1 molar equivalents of PEG:S-Fab). After shaking at 22°C for 2 h, the samples were examined by reducing SDS-PAGE (5 µL per sample). (**A**) Coomassie blue staining of S-Fab with different molar ratios of PEG:S-Fab during PEGylation. (**B**) Barium iodide complex dye staining for S-Fab with different molar ratios of PEG:S-Fab. (**C**) Western blotting assay using an antiflag antibody to detect the VH-CH-CEA chain. (**D**) Western blotting assay using an anti-His6 antibody to detect the VL-CL chain. Numbers 0, 10, 20, 40, and 60 represent the molar ratio of PEG:Fab at 0:1, 10:1, 20:1, 40:1, and 60:1, respectively. M (kDa), molecular weight markers (kilodalton).

Abbreviations: CEA, carcinoembryonic antigen; MAL-PEG-OMe, methoxy PEG maleimide; PEG, polyethylene glycol; S-Fab, single-domain antibody-linked Fab; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

3)Purification of PEG-S-Fab using fast

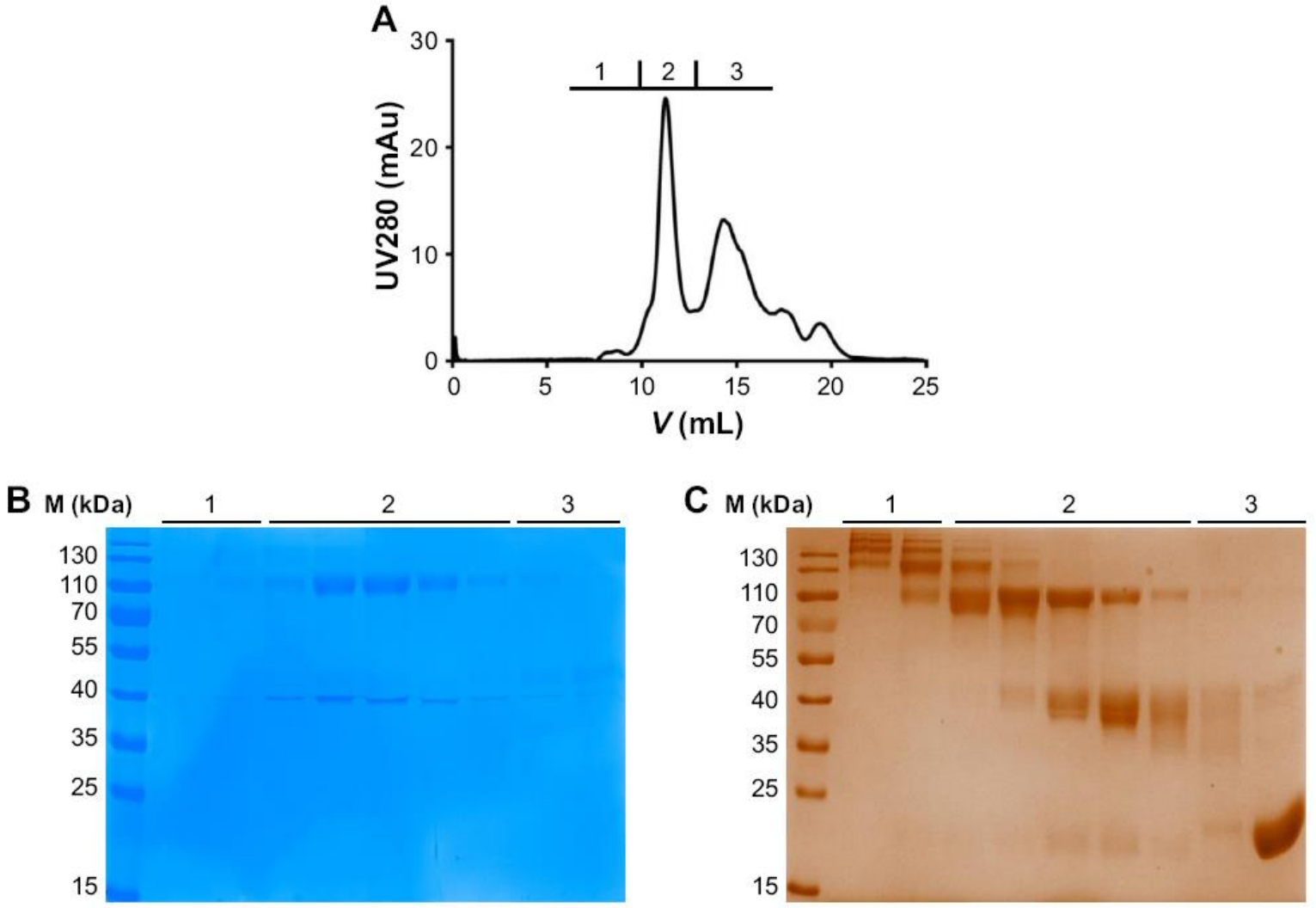
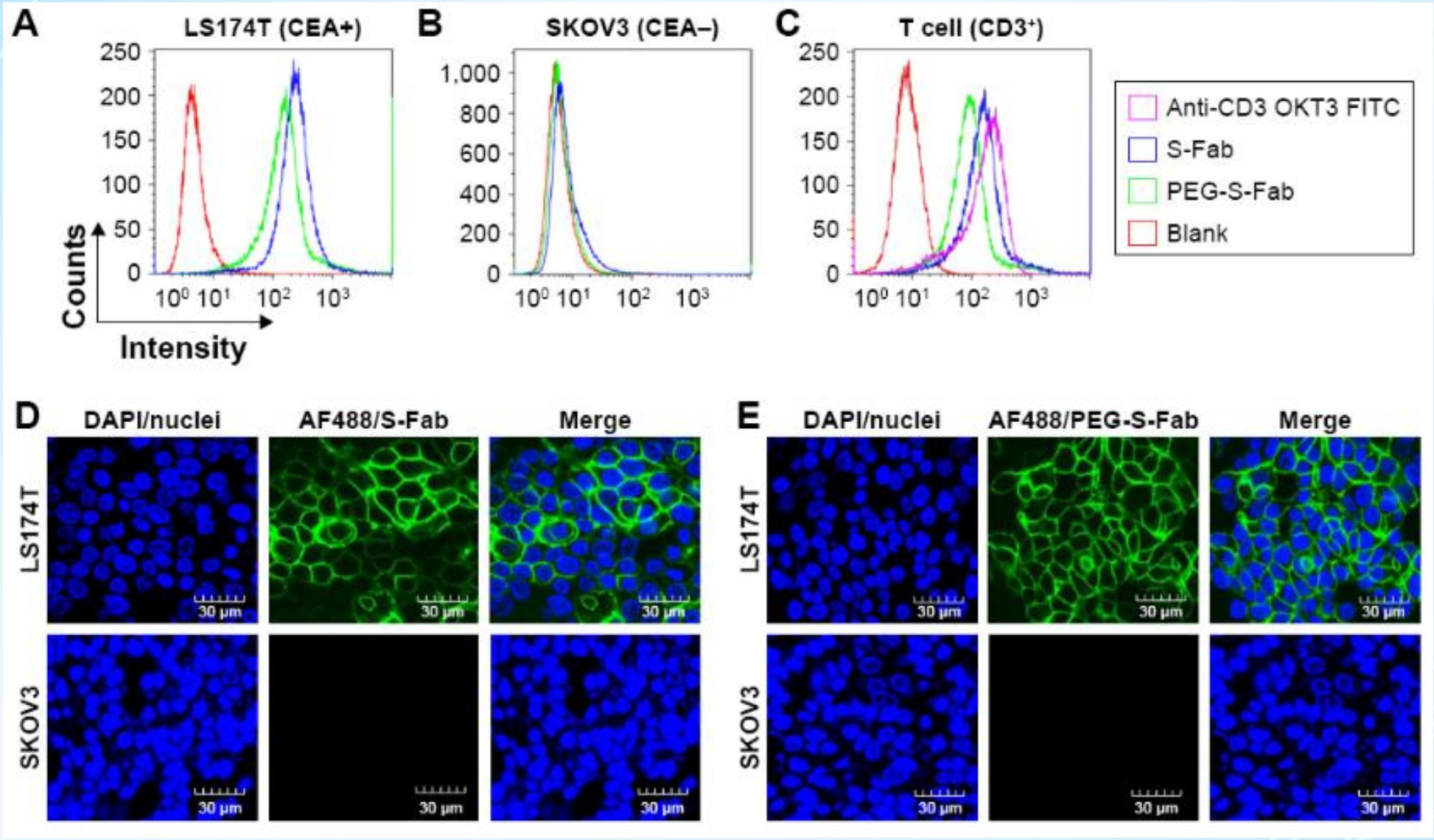


Figure 3 Purification of PEG-S-Fab.

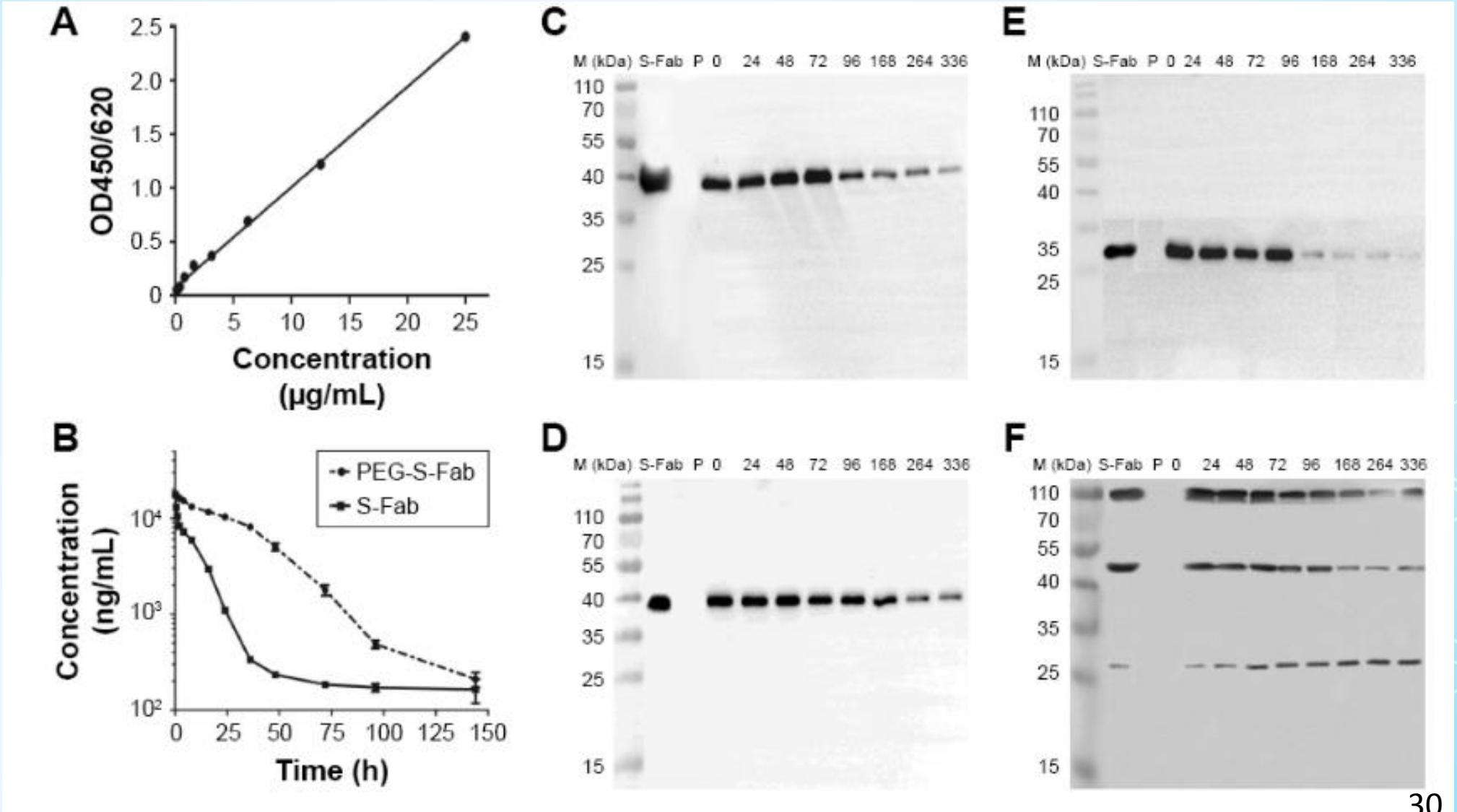
Notes: Gel filtration was used to fractionate the PEGylation mixture (fractions 1, 2, and 3). (**A**) Chromatogram of PEG-S-Fab. (**B**) Coomassie blue staining of the fractions 1, 2, and 3 from PEG-S-Fab purification. (**C**) Barium iodide complex staining of the fractions 1, 2, and 3 from PEG-S-Fab purification. M (kDa), molecular weight markers (kilodalton).

Abbreviations: PEG, polyethylene glycol; PEG-S-Fab, PEGylated S-Fab; S-Fab, single-domain antibody-linked Fab; UV, ultraviolet.

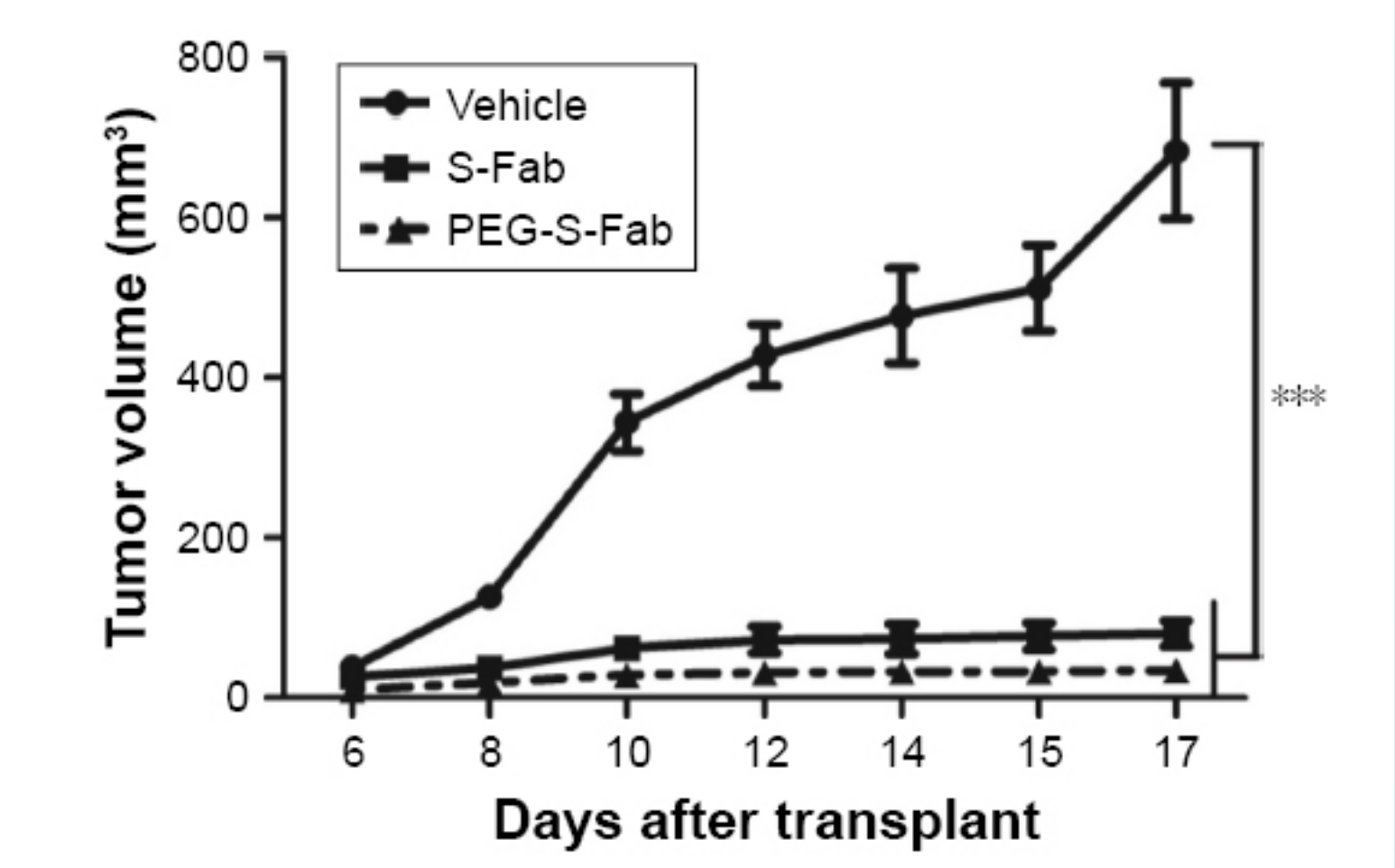
4)PEG-S-Fab can bind the tumor antigen CEA and CD3 T cells



6)PEGylation prolongs the in vivo half-life of s-fab



7)PEG-S-fab induces more potent in vivo antitumor activity



Discussion

- In this study, we demonstrated that the site-specific PEGy-lation can improve the therapeutic profiles of the bispecific
- S-Fab showed potent antitumor activities in vitro and in vivo
- As a Fab format, S-Fab offers advantages over full-length antibodies, including better penetration into tumor tissues and simple production of the antibody using inexpensive prokaryotic expression systems.
- the short in vivo half-life of S-Fab limits its potential as a therapeutic agent.

In the case of antibody fragments, PEGylation has been shown to lengthen their circulating half-life, enhance their proteolytic resistance of therapeutic proteins, and reduce their immunogenicity.

PEGylation potentially decrease enzymatic proteins or the binding affinities of Fabs.

Flow cytometry analysis showed that PEGylation had a slight effect on the binding of S-Fab to tumor cells and T cells and slightly reduced the in vitro cytotoxicity

PEGylation increased the circulation t 1/2 of S-Fab by 12-fold in vivo

. enhancement of the in vivo half was due to the hydrodynamic radius and enhanced molecular size of the PEGylated conjugate
leading to reduced renal clearance and increased resistance of the PEGylated conjugate to proteolysis.

In mouse models, PEG-S-Fab showed more potent antitumor activity than S-Fab

Conclusion

this study supports the use of PEGylation to extend the circulating half-life of the bispecific antibody
S-Fab and enhance its therapeutic potential in the clinic.
This strategy can be potentially applied to a broad range of immunotherapies.

با تشکر از توجه شما